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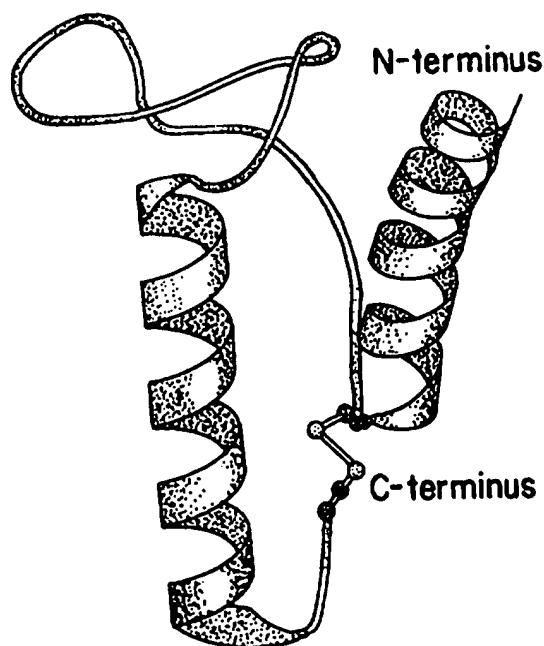
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(54) Title: POLYPEPTIDE FRAGMENTS DERIVED FROM THE OBESE GENE PRODUCT

(57) Abstract

Novel C-terminal fragments of the *obese* gene product. The fragments retain anti-obesity and/or anti-diabetic activities. The fragments contain less than 68 amino acids, two of which are capable of forming cross-linkages, preferably cysteines.



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POLYPEPTIDE FRAGMENTS DERIVED FROM THE OBESE GENE PRODUCT

The present invention concerns polypeptide fragments derived from the obese (*ob*) gene product and uses thereof.

5 The mouse and human *ob* gene were recently identified by positional cloning and sequencing (Friedman et al. Nature 372, 425-432 (1994)). *ob* encodes a 4.5 kb adipose tissue messenger RNA containing a predicted 167-amino acid open reading frame (ORF). The predicted amino acid sequence is 84% identical between human and mouse and has features of a secreted protein. The extensive homology
10 deduced for the *ob* gene product among vertebrates suggests that its function is highly conserved. On the basis of their genetic studies, Friedman and coworkers have speculated that the *ob* gene product may function as part of a signalling pathway from adipose tissue acting to regulate the size of the body fat depot.

Preparation of the *ob* protein with high specific activity by a number of
15 recombinant methods has proven difficult [Pelleymounter et al. Science 269, 540-543 (1995); Halaas et al. *ibid.* 543-546].

It has been discovered that small polypeptide fragments of the *ob* gene product can be facilely prepared, retain biological activity, and overcome some of the drawbacks of the full length protein described in the prior art.

20 The present invention is directed to polypeptide fragments of the *obese* gene product which have anti-obesity and/or anti-diabetic activity. These polypeptides will be referred to herein as the active *obese* gene product fragments or "OBF" which will be further defined hereinafter. The invention also includes therapeutic methods using OBF.

25 Accordingly, in a first embodiment, the invention is directed to purified preparations of OBF. A preferred murine OBF has the sequence of SEQ.ID.NO.:1 as follows:

30 Leu-Glu-Asn-Leu-Arg-Asp-Leu-Leu-His-Leu-Leu-Ala-Phe-Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gln-Thr-Ser-Gly-Leu-Gln-Lys-Pro-Glu-Ser-Leu-Asp-Gly-Val-Leu-Glu-Ala-Ser-Leu-Tyr-Ser-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Gln-Gly-Ser-Leu-Gln-Asp-Ile-Leu-Gln-Gln-Leu-Asp-Val-Ser-Pro-Glu-Cys, S,S-crosslinked

A preferred human OBF has the sequence of SEQ.ID.NO.:2 as follows:

Leu-Glu-Asn-Leu-Arg-Asp-Leu-Leu-His-Val-Leu-Ala-Phe-Ser-Lys-Ser-Cys-His-Leu-
Pro-Trp-Ala-Ser-Gly-Leu-Glu-Thr-Leu-Asp-Ser-Leu-Gly-Gly-Val-Leu-Glu-Ala-Ser-
Gly-Tyr-Ser-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Gln-Gly-Ser-Leu-Gln-Asp-Met-
5 Leu-Trp-Gln-Leu-Asp-Leu-Ser-Pro-Gly-Cys, S,S-crosslinked

Another preferred murine OBF has the sequence of SEQ.ID.NO.:3 as follows:

Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gln-Thr-Ser-Gly-Leu-Gln-Lys-Pro-Glu-Ser-Leu-Asp-
Gly-Val-Leu-Glu-Ala-Ser-Leu-Tyr-Ser-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Gln-
10 Gly-Ser-Leu-Gln-Asp-Ile-Leu-Gln-Gln-Leu-Asp-Val-Ser-Pro-Glu-Cys, S,S-crosslinked

Another preferred human OBF has the sequence of SEQ.ID.NO.:4 as follows:

Ser-Lys-Ser-Cys-His-Leu-Pro-Trp-Ala-Ser-Gly-Leu-Glu-Thr-Leu-Asp-Ser-Leu-Gly-
Gly-Val-Leu-Glu-Ala-Ser-Gly-Tyr-Ser-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Gln-
15 Gly-Ser-Leu-Gln-Asp-Met-Leu-Trp-Gln-Leu-Asp-Leu-Ser-Pro-Gly-Cys, S,S-
crosslinked

In a further embodiment, the invention is directed to a method for treating obesity in a patient, wherein the obesity is related to a defect in regulation by the *obese* protein or can otherwise be overcome by elevating *obese* protein levels, the
20 method comprising administering an effective amount of OBF to the patient.

In a further embodiment, the invention is directed to a method for treating diabetes in a patient in need of treatment, where the diabetes is *obese* protein - associated, the method comprising administering an effective amount of OBF to the patient.

25 In a further embodiment, the invention is directed to a method for diagnosing *obese* protein-associated diabetes or obesity.

In a further embodiment, the invention is directed to an isolated nucleic acid sequence encoding OBF.

30 In a further embodiment, the invention is directed to a vector containing nucleic acid encoding OBF.

In a further embodiment, the invention is directed to a host cell transformed with the vector of the invention.

In a further embodiment, the invention is directed to a method for producing OBF from the recombinant host.

35 Figure 1. Three dimensional representation of preferred OBF having 67 amino acids and three alpha helices.

It has been discovered that fragments of the *ob* protein retaining biological activity and accessible by chemical synthesis overcome some of the drawbacks of the full length protein. Specifically, it has been found by secondary structure analysis and molecular modeling that the C-terminal peptide fragments containing the only two
5 cysteine residues present in the mature *ob* protein, which are preferably disulfide-linked (or functionally equivalent residues that are capable of forming cross-linkages), retains the conformation and overall folding needed for biological activity.

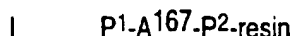
As used herein, the following terms have the indicated meanings: "therapeutic methods" means the anti-obesity and/or the anti-diabetic methods of the invention;
10 "OBF" means the optionally mutated or modified biologically active C-terminal fragments of the *ob* gene product having less than sixty-eight amino acids and containing two residues at positions 117 and 167 that are capable of forming cross-linkages, preferably cysteine residues; "obese protein-associated" means that the obesity and/or diabetes is partially or wholly due to mutations of the *ob* gene or any
15 alteration or defect in the *ob* gene product or metabolic pathway(s) involving the *ob* gene product resulting in increased risk or clinical manifestation of obesity and/or diabetes; "biologically active" means having serum glucose lowering activity useful for treating type II diabetes; or serum triglyceride lowering activity, or decreased body weight gain, or decreased body weight, or reduction in food consumption or appetite
20 useful for treating obesity.

OBF preferably has 67 amino acids and most preferably has the amino acid sequence identified as SEQ. ID. NO:1 or SEQ.ID.NO.:2; however, other biologically active C-terminal fragments of the *ob* gene product having less than 68 amino acids are also specifically contemplated to be within the scope of the present invention.
25 The amino acid numbering scheme used herein is based on the intact *ob* protein and is the same as described by Friedman and coworkers in Nature 372, 425-432 (1994). Such fragments of the invention, which include mutants or modified fragments that retain biological activity, contain amino acid sequences with the following typical features: (1) residues in positions 117 and 167 with side-chains capable of forming
30 cross-linkages such as disulfide, lactam, lactone, dicarba-cystine and the like; (2) an amphipathic alpha-helix spanning about 15-25 C-terminal residues; (3) a preferred but optional amphipathic alpha-helix N-terminal to the Cys 117 or equivalent residue, spanning about 15-25 residues; (4) a loop supported by at least one helical scaffold and at least one covalent cross-linkage. OBF containing one additional cross-linkage
35 between the C-terminal alpha-helix and either the loop or the N-terminal alpha-helix, if present are specifically contemplated to be within the scope of the present invention.

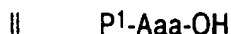
The present invention specifically contemplates polypeptides that are at least 30% homologous to the polypeptides of SEQ.ID.NO.:1, SEQ.ID.NO.:2, SEQ.ID.NO.:3, or SEQ.ID.NO.:4, preferably at least 50% homologous, more preferably at least 80% homologous, and most preferably at least 90% homologous. Residues 117 and 167 are preferably cross-linked via covalent bonds such as disulfide, lactam, lactone, dicarba-cystine and the like. The OBF preferably has cysteine residues at positions 117 and 167 which are preferably cross-linked via disulfide bonds. In addition, the N-terminus of such fragments is typically blocked with an acetyl or similar N-blocking group.

10 OBF can be prepared by chemical synthesis using the following exemplary process:

A resin-coupled carboxyl terminal residue (I)



15 is treated with a deprotecting agent (e.g., piperidine) in an organic solvent such as N-methylpyrrolidinone (NMP) or dimethylformamide (DMF); where A^{167} is Cys or equivalent residue. The resulting deprotected peptidyl-resin is coupled with the N- α -protected amino acid (II)



20 in the presence of a coupling reagent such as 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) in NMP and diisopropylethylamine (DIEA) at about 20 to 30 °C in a molar ratio of about 1: 5 compound I:II to form a resin-coupled dipeptide (III)



30 In compounds I through III and throughout this specification, P^1 is an amino-protecting group such as fluorenylmethoxycarbonyl (Fmoc) and P^2 is a *para*-alkoxybenzyl ester linkage. The reaction may be effected with an automated peptide synthesizer (e.g., Applied Biosystems 431A or 433A.). The foregoing procedure for peptide III is repeated with other nitrogen-protected and side-chain protected amino acids until all
35 desired residues, e.g., of SEQ. ID. NO. 1 or SEQ. ID. NO. 2, have been coupled to the resin. Sidechain-protecting groups may be used in this process for sidechains having

reactive functionalities, such as hydroxyl, carboxyl, amino, mercapto, guanidino, imidazolyl, indolyl and the like. The particular protecting groups used for any amino acid residues depend upon the sidechains to be protected and are generally in the art. Exemplary sidechain protecting groups are *t*-butyl, benzyl, benzoyl, acetyl, 5 halocarbobenzoxy, and the like for hydroxyl; cyclohexyl, benzyl, methyl, ethyl, *t*-butyl and the like for carboxyl; benzyl, 4-methylbenzyl, 4-methoxybenzyl, acetyl, acetamidomethyl, triphenylmethyl (trityl), and the like for mercapto; *t*-butyloxycarbonyl (Boc), carbobenzoxy (Cbz), halocarbobenzoxy, 9-flourenylmethoxycarbonyl (Fmoc), phthaloyl (Pht), *p*-toluenesulfonyl (Tos), 10 trifluoroacetyl, 2-(trimethylsilyl)ethoxycarbonyl (Teoc), and the like for amino; 2,4-dinitrophenyl, benzyloxymethyl, Tos, Boc, trityl, and the like for imidazolyl; Cbz, Teoc, 2,2,2-trichloroethyl carbamate (Troc), formyl, and the like, or no protection, for indolyl; and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc), 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr), Tos, nitro, bis-(1-adamantyloxycarbonyl) and the like 15 for guanidino.

Side chain protecting groups may be removed, if desired, by treatment with one or more deprotecting agents in an inert solvent or solvent mixture (e.g., NMP or DMF). Suitable deprotecting agents are generally known in the art. Exemplary deprotecting agents are thiophenol, mercaptoethanol and the like for removing 2,4- 20 dinitrophenyl; trifluoroacetic acid (TFA) and the like for butoxycarbonyl; hydrofluoric acid (HF), trifluoromethanesulfonic acid (TFMSA) and the like for several different protecting groups. For further examples of protecting groups and suitable deprotecting agents, see Bodansky, M. and Bodansky, A., The Practice of Peptide Synthesis, Springer-Verlag, Inc. (1984); and Greene, T. W. and Wuts, P., Protective Groups in Organic Synthesis (2nd ed.), John Wiley & Sons, Inc. (1991). 25

The fully formed polypeptide may be cleaved from the resin by methods generally known in the art. For example, the resin-bound polypeptide may be treated with an acid, such as TFA, HF, TFMSA, and the like.

Cross-linkage of the two cysteine residues via disulfide bond may be effected 30 by methods generally known in the art. For example, the deprotected peptide may be stirred overnight in 0.1 M ammonium bicarbonate (pH 7.8) or in dilute ammonium hydroxide (pH 8.0-8.5) in the presence or not of 10% dimethylsulfoxide.

Purification of the OBF can be effected by methods generally known in the art. For example, the crude peptide may be dissolved in a solution of 35 water/acetonitrile/TFA (60:40:0.1, v:v:v) and loaded onto a C18 reverse phase

column. A gradient of acetonitrile in water, both buffered with 0.1% TFA, can then be used to elute the desired OBF.

OBF also can be prepared by use of genetic engineering techniques. The process for genetically engineering the OBF coding sequence, for expression under a desired promoter, is facilitated through the cloning of genetic sequences which are capable of encoding OBF. These cloning technologies can utilize techniques known in the art for construction of a DNA sequence encoding OBF, such as polymerase chain reaction technologies utilizing a OBF sequence to isolate the *ob* gene *de novo*, or polynucleotide synthesis methods to construct the nucleotide sequence using chemical methods. Expression of the cloned OBF DNA provides OBF.

As used herein, the term "genetic sequences" is intended to refer to a nucleic acid molecule (preferably DNA). Genetic sequences that are capable of being operably linked to DNA encoding OBF, so as to provide for its expression and maintenance in a host cell, are obtained from a variety of sources, including commercial sources, genomic DNA, cDNA, synthetic DNA, and combinations thereof. Since the genetic code is universal, it is to be expected that any DNA encoding the OBF amino acid sequence of the invention will be useful to express OBF in any host, including prokaryotic (bacterial) and eukaryotic (plants, mammals (especially human), insects, yeast, and especially cultured cell populations).

20

An example of mouse DNA sequence encoding OBF is SEQ.ID.NO.:5 as follows:

CCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGA
GCTGCTCCGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGA
TGGCGTCCTGGAAGCCTCACTCTACTCCACAGAGGTGGTGGCTTTGA
GCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACAGTTGGATGTT
AGCCCTGAATG

Another example of mouse DNA sequence encoding OBF is SEQ.ID.NO.:6 as follows:
CTCCAAGAGCTGCTCCGCCTCAGACCAGTGGCCTGCAGAAGCCAGAG
AGCCTGGATGGCGTCCTGGAAGCCTCACTCTACTCCACAGAGGTGGT
GGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACAGT
TGGATGTTAGCCCTGAATG

An example of human DNA sequence encoding OBF is SEQ.ID.NO.:7 as follows:

CTGGAGAACCTCCGGGATCTTCTTCACGTGCTGGCCTTCT
 CTAAGAGCTGCCACTTGCCCTGGGCCAGTGGCCTGGAGACCTTGGAC
 AGCCTGGGGGGTGTCTGGAAGCTTCAGGCTACTCCACAGAGGTGGT
 5 GGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATGCTGTGGCAGC
 TGGACCTCAGCCCTGGGTGC

Another example of human DNA sequence encoding OBF is SEQ.ID.NO.:8 as follows:

TCTAAGAGCTGCCACTTGCCCTGGGCCAGTGGCCTGGAGACCTTGGG
 10 CAGCCTGGGGGGTGTCTGGAAGCTTCAGGCTACTCCACAGAGGTGG
 TGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATGCTGTGGCAG
 CTGGACCTCAGCCCTGGGTGC

Due to the degeneracy of the genetic code, other DNA sequences which
 15 encode the same amino acid sequences of SEQ.ID.NOS.: 1,2,3, and 4 may be used for
 the production of the OBF of the present invention. In addition, it will be understood
 that allelic variations of these DNA and amino acid sequences naturally exist, of
 may be intentionally introduced using methods known in the art. These variations
 may be demonstrated by one or more amino acid differences in overall sequence,
 20 or by deletions, substitutions, insertions, inversions or additions of one or more amino
 acids in said sequence. Such amino acid substitutions may be made, for example, on
 the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity
 and/or the amphipathic nature of the residues involved. For example, negatively
 charged amino acids include aspartic acid and glutamic acid; positively charged amino
 25 acids include lysine and arginine; amino acids with uncharged polar head groups or
 nonpolar head groups having similar hydrophilicity values include the following: leucine,
 isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine;
 phenylalanine, tyrosine. Other contemplated variations include salts and esters of
 the aforementioned polypeptides, as well as precursors of the aforementioned
 30 polypeptides, for example, precursors having N-terminal substitutions such as
 methionine, N-formylmethionine and leader sequences. All such variations are
 included within the scope of the present invention.

It is specifically contemplated that the present invention include DNA
 sequences that code for biologically active polypeptides of less than 68 amino acids
 35 and that are capable of hybridizing under stringent conditions to a sequence
 complementary to SEQ.ID.NOS.: 5, 6, 7, or 8. Stringent hybridization conditions select

for DNA sequences of greater than 30% homology, preferably greater than 85% or, more preferably, greater than 90% homology. Screening DNA under stringent conditions may be carried out according to the method described in Nature, 313: 402-404 (1985). The DNA sequences capable of hybridizing under stringent conditions with the DNA disclosed in the present application may be, for example, allelic variants of the disclosed DNA sequences, may be naturally present in the human or mouse but related to the disclosed DNA sequences, or may be derived from other animal sources. General techniques of nucleic acid hybridization are disclosed by Maniatis, T. et al., In: Molecular Cloning, a Laboratory Manual, Cold Spring Harbor, N.Y. (1982), and by Haymes, B.D. et al., In: Nucleic Acid Hybridization, a Practical Approach, IRL Press, Washington, D.C. (1985), which references are incorporated herein by reference.

When the OBF DNA coding sequence and an operably linked promoter are introduced into a recipient eukaryotic cell (preferably a human host cell) as a non-replicating, non-integrating, molecule, the expression of the encoded OBF can occur through the transient (nonstable) expression of the introduced sequence.

Preferably the coding sequence is introduced on a DNA molecule, such as a closed circular or linear molecular that is capable of autonomous replication. If integration into the host chromosome is desired, it is preferable to use a linear molecule. If stable maintenance of OBF DNA is desired on an extrachromosomal element, then it is preferable to use a circular plasmid form, with appropriate plasmid element for autonomous replication in the desired host.

The desired gene construct, providing DNA coding for OBF, and the necessary regulatory elements operably linked thereto, can be introduced into desired host cells by transformation, transfection, or any method capable of providing the construct to the cell. A marker gene for the detection of a host cell that has accepted the OBF DNA can be on the same vector as the OBF DNA or on a separate construct for co-transformation with the OBF coding sequence construct into the host cell. The nature of the vector will depend on the host organism.

Suitable selection markers will depend upon the host cell. For example, the marker can provide biocide resistance, e.g, resistance to antibiotics, or heavy metals, such as copper, or the like.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector can be recognized and selected from those recipient cells which do not contain the vector; the number of

copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells or different species.

Oligonucleotide probes specific for the OBF sequence can be used to identify clones to OBF and can be designated *de novo* from the knowledge of the amino acid sequence of the protein as provided herein in SEQ.ID.NO.:1 or from the knowledge of the nucleic acid sequence of the DNA encoding such protein or of a related protein. Alternatively, antibodies can be raised against OBF and used to identify the presence of unique protein determinants in transformants that express the desired cloned polypeptide.

10 A nucleic acid molecule, such as DNA, is said to be "capable of expressing" OBF if that nucleic acid contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the OBF nucleotide sequence which encode the OBF polypeptide.

An operable linkage is a linkage in which a sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the sequence under the influence or control of the regulatory sequence. If the two DNA sequences are a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence, they are operably linked if induction of promoter function results in the transcription of mRNA encoding the desired protein and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the protein, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region is operably linked to a DNA sequence if the promoter is capable of effecting transcription of that DNA sequence.

25 The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but includes, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

35 The vectors of the invention can further comprise other preferably linked regulatory elements such as DNA elements which confer antibiotic resistance, or origins of replication for maintenance of the vector in one or more host cells.

Expression of a protein in eukaryotic hosts such as a human cell requires the use of regulatory regions functional in such hosts. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the host. Preferably, these regulatory signals are associated in their native state with a particular gene which is capable of a high level of expression in the specific host cell, such as specific human tissue type. In eukaryotes, where transcription is not linked to translation, such control regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell.

If desired, the non-transcribed and/or non-translated regions 3' to the sequence coding for *ob* protein can be obtained by the above-described cloning methods. The 3'-non-transcribed region of the native human *ob* gene can be retained for its transcriptional termination regulatory sequence elements, or for those elements which direct polyadenylation in eukaryotic cells. Where the native expression control sequences signals do not function satisfactorily in a host cell, sequences functional in the host cell can be substituted.

It may be desired to construct a fusion production that contains a partial coding sequence (usually at the amino terminal end) of a first protein or small peptide and a second coding sequence (partial or complete) of OBF at the carboxyl end. The coding sequence of the first protein can, for example, function as a signal sequence for secretion of OBF from the host cell. Such first protein can also provide for tissue targeting or localization of OBF if it is to be made in one cell type in a multicellular organism and delivered to another cell type in the same organism. Such fusion protein sequences can be designed with or without specific protease sites such that a desired peptide sequence is amenable to subsequent removal.

The expressed OBF can be isolated and purified from the medium of the host in accordance with conventional conditions, such as extraction, precipitation, ion exchange chromatography, size exclusion chromatography, affinity chromatography, high performance liquid chromatography on reverse phase, electrophoresis, or the like. For example, affinity purification with anti-OBF antibody can be used. A polypeptide having the amino acid sequence of, for example, SEQ.ID.NO.:1 or SEQ.ID.NO.:2 can be made, or a shortened peptide of either of these sequences can be made, and used to raise antibodies using methods well known in the art. These antibodies can be used to affinity purify or quantitate OBF from any desired source.

If it is necessary to extract OBF from the intracellular regions of the host cells, the host cells can be collected by centrifugation, or with suitable buffers, lysed, and the protein isolated by the column chromatography, for example, on DEAE-cellulose, phosphocellulose, polyribocytidylc acid-agarose, hydroxyapatite or by electrophoresis or immunoprecipitation.

It is to be understood that all of the above procedures that are applicable to cloning and expressing OBF sequences apply equally to normal and mutant sequences. Mutations may be in any of the regions, i.e., coding, non-coding, exonic, intronic, regulatory and the like.

Mouse *ob* coding sequence can be amplified using the polymerase chain reaction (PCR) from mouse adipose tissue cDNA using synthetic oligonucleotides corresponding to the 5' and 3' ends of the 501 nucleotide coding sequence (Friedman et al. Nature 372, 425-432 (1994)), and cloned into the *E. coli* expression vector pGEX-2T (Pharmacia) to produce a C-terminal glutathione-S-transferase fusion protein coding sequence. Further constructs could be made that would allow heterologous *ob* protein expression in baculovirus-infected insect cells, COS cells, 293 cells, and *Saccaromyces cerevisia* by cloning the above *ob* coding sequence into the vectors pVL1393, pCDNA1/amp, pCEP4 β , and a suitable *Saccaromyces cerevisia* vector, respectively.

It is understood that although the following discussion is specifically directed to human patients, the teachings are also applicable to any animal which would benefit from practice of the methods of the invention.

In the methods of treating obesity and/or diabetes in a patient in need of such treatment, OBF, preferably of the formula SEQ. ID. NOS.: 1, 2, 3, or 4, may be used to reduce serum glucose and/or triglyceride levels in obese patients and type II diabetics. Plasma glucose and triglyceride levels are significantly decreased in *ob/ob* mice, following infusion with OBF, relative to infusion with a control peptide. The anti-diabetic effect is independent of an effect on food consumption.

In the therapeutic methods of the invention, the form of administration of OBF can be any form known in the pharmaceutical art. The amount of OBF to be administered would depend in part on the age, weight, and general condition of the patient. Typically, a patient would be closely monitored by a physician who would determine if the dosage amount or regimen of OBF being administered was effective and well tolerated. OBF would be administered either alone or admixed with a pharmaceutically acceptable carrier. Administration can be parenteral or enteral depending upon the dosage form and the needs of the patient.

The effective amount of OBF in either the anti-obesity or anti-diabetic methods of the invention is typically about 0.001 to about 1000 milligrams (mgs) per kilogram (kg) of body weight (wt) per day, preferably about 0.05 to about 50 mgs per kg of body weight per day.

5 It is another advantage of the present invention that activity-retaining radioisotopes of OBF are easier to prepare than those of corresponding full length protein. For example, the OBF polypeptide defined by SEQ. ID. NOS.:1, 2, 3, or 4 may be labeled with a radioisotope including radioactive iodo (^{125}I) or hydrogen (^3H) atoms. Alternatively, labeling of the OBF polypeptide defined by SEQ. ID. NOS.:1, 2,
10 3, or 4 with radioactive iodo (^{125}I) atoms may be achieved by reaction with prelabeled Bolton-Hunter reagent. Thus, by monitoring of the radioactive polypeptide, cells could be diagnosed for the presence of cellular components that bind the polypeptide. Furthermore, a radiolabelled OBF (e.g., the polypeptide defined by SEQ. ID. NOS.: 1, 2, 3, or 4) is useful for the cloning and expression of the *ob* protein
15 receptor. Accordingly, the present invention is also directed to a method of diagnosing obesity or diabetes or a predisposition to develop obesity or diabetes in an individual, said method comprising

- (a) labelling OBF;
- (b) contacting the labelled OBF with biological material from said
20 individual;
- (c) monitoring the biological material for the presence of bound OBF to obtain a first set of results;
- (d) comparing the first set of results from step (c) with with a second set of results obtained using similar monitoring in an
25 individual not having OBF-associated obesity or diabetes; and
- (e) diagnosing the presence of obesity or diabetes in said individual or the tendency of said individual to develop obesity or diabetes by comparing the first set of results and second set of results to determine if the results differ.

30 The labelling can be any type of labelling known in the art such as radiolabelling, fluorescent labelling, biotinylation, and the like.

OBF of the present invention may also be used to prepare in a known manner polyclonal or monoclonal antibodies capable of binding the OBF. These antibodies may in turn be used for the detection of the *ob* protein in a sample, for
35 example, a plasma sample or cell sample, using immunoassay techniques, for example, radioimmunoassay or enzyme immunoassay. The antibodies may also be

used in affinity chromatography for purifying OBF or *ob* protein and isolating it from various sources. Thus the present invention is also directed to a method of diagnosing obesity or diabetes or a predisposition to develop obesity or diabetes in an individual, said method comprising

- 5 (a) raising antibodies to OBF in a suitable mammal such as mouse, rabbit, horse, etc.,
- (b) contacting said antibodies with a test biological sample and allowing the antibody to bind to the sample;
- (c) measuring the amount of antibody bound to the sample;
- 10 (d) repeating steps (b) and (c) using a standard having a known affinity to the antibodies instead of the test sample;
- (e) comparing the results using the test sample with the results using the standard to determine the amount of antibodies bound to the test sample relative to the amount of antibodies bound to the standard.
- 15

The manner and method of carrying out the present invention can be more fully understood by those of skill by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

20

Example 1 Process of Preparation

SEQ. ID. NO.:1

25 N-Acetyl-L-leucyl-L- α -glutamyl-L-asparaginy-L-leucyl-L-arginyl-L- α -aspartyl-L-leucyl-L-leucyl-L-histidyl-L-leucyl-L-leucyl-L-alanyl-L-phenylalanyl-L-seryl-L-lysyl-L-seryl-L-cysteinyl-L-seryl-L-leucyl-L-prolyl-L-glutaminy-L-threonyl-L-serylglycyl-L-leucyl-L-glutaminy-L-lysyl-L-prolyl-L- α -glutamyl-L-seryl-L-leucyl-L- α -aspartylglycyl-L-valyl-L-leucyl-L- α -glutamyl-L-alanyl-L-seryl-L-leucyl-L-tyrosyl-L-seryl-L-threonyl-L-
30 α -glutamyl-L-valyl-L-valyl-L-alanyl-L-leucyl-L-seryl-L-arginyl-L-leucyl-L-glutaminyglycyl-L-seryl-L-leucyl-L-glutaminy-L- α -aspartyl-L-isoleucyl-L-leucyl-L-glutaminy-L-glutaminy-L-leucyl-L- α -aspartyl-L-valyl-L-seryl-L-prolyl-L- α -glutamyl-L-cysteine, S,S-crosslinked

A protected peptide ("intermediate A") of SEQ.ID.NO.:1 was assembled
35 stepwise by the solid phase method on an Applied Biosystems Model 431A Peptide Synthesizer using the Fmoc/HBTU/HOBt program supplied with the instrument. The starting Fmoc-Cys(Trt)-HMP resin (polystyrene, 1% divinylbenzene copolymer)

- was purchased from Midwest Bio-Tech (g, 0. mmol). Amino acids were coupled as their N α -Fmoc derivatives. The side chains of Ser, Thr and Tyr were protected with the t-butyl group; those of Cys, Asn, His and Gln with the trityl group, those of Asp and Glu with the t-butyl ester group, that of Arg with the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC) group and that of Lys with the N ϵ -t-butyloxycarbonyl (t-Boc) group. Fmoc groups were removed at each cycle by a 6 min treatment with 20% piperidine in NMP. Each amino acid derivative was double coupled (25 min, room temperature), in 5-fold molar excess, using one equivalent of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) in NMP and DIEA. After the final coupling, the N-terminal Fmoc group was removed as described above and the resulting peptidyl-resin was washed with NMP and treated twice with 1-acetyl imidazole (550 mg, 5.0 mmol) in NMP (8 mL) for 25 min. The resulting 67 amino acid peptidyl-resin was washed with NMP and DCM and dried, yielding 1.095 g of A.
- 15 Simultaneous deprotection and cleavage of the peptide (intermediate A) from the resin was accomplished by stirring the protected polypeptidyl-resin (1.095 g, 0.083 mmol) in 22 mL of CF₃COOH/water/thioanisole/dithiothreitol/phenol (10:0.5:0.6:1.0:0.8, v/v/v/w/w) for 3 hours at room temperature. The resin was removed by filtration and the filtrate was concentrated in vacuo. The crude product was precipitated with cold methyl t-butyl ether (MTBE), filtered and washed with MTBE, then redissolved in 0.1% aqueous NH₄OH and lyophilized. Yield: 0.545 g (83% of theory).
- 20 A portion of the crude linear product (120 mg) was stirred in 66 mL of 0.1 M NH₄HCO₃/DMSO (9:1, v:v) for 36 hours at room temperature. The solution was lyophilized to yield a solid. Five sample aliquots (20 mg each) were injected into a Vydac C18 column (22X250 mm, 10 μ , 300 Å) and reverse phase HPLC was performed under the following conditions: Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in CH₃CN; linear gradient from 43 to 48% B in A over 50 min; flow rate: 12 mL/min. The fractions containing the major peak (215 nm) eluting between 37.0 and 38.5 min were pooled and lyophilized to yield 1.2 mg (1.2%) of Example 1 as a white, fluffy solid.
- 30 Analytical HPLC of the above-prepared OBF: Vydac C18 (4.6 x 250 mm); 215 nm, 1.0 mL/min: linear gradient from 40% to 60% B in A over 50 minutes; retention time: 20.36 minutes. Purity: greater than 95% (Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in acetonitrile). MS (electrospray): found molecular weight (derived) 7338.09. Amino acid analysis: Asx(5) 4.1; Glx(11) 12.6; Ser(10) 8.7; Gly (3) 5.1;

His(1) 0.5; Arg(2) 1.7; Thr(2) 1.7; Ala(3) 3.2; Pro(3) 5.0; Tyr(1) 0.6; Val(4) 3.8; Ile(1) 1.2; Leu(16) 15.0; Phe(1) 0.7; Lys(2) 1.6; Cys(2) not detected.

Example 2

SEQ. ID. NO.:3

5

N-Acetyl-L-seryl-L-lysyl-L-seryl-L-cysteinyl-L-seryl-L-leucyl-L-prolyl-L-glutamyl-L-threonyl-L-serylglycyl-L-leucyl-L-glutamyl-L-lysyl-L-prolyl-L- α -glutamyl-L-seryl-L-leucyl-L- α -aspartylglycyl-L-valyl-L-leucyl-L- α -glutamyl-L-alanyl-L-seryl-L-leucyl-L-tyrosyl-L-seryl-L-threonyl-L- α -glutamyl-L-valyl-L-valyl-L-alanyl-L-leucyl-L-seryl-L-arginyll-L-leucyl-L-glutamylglycyl-L-seryl-L-leucyl-L-glutamyl-L- α -aspartyl-L-isoleucyl-L-leucyl-L-glutamyl-L-glutamyl-L-leucyl-L- α -aspartyl-L-valyl-L-seryl-L-prolyl-L- α -glutamyl-L-cysteine, S,S-crosslinked

10

A protected peptide ("intermediate B") of SEQ.ID.NO.:3 was assembled stepwise by the solid phase method on an Applied Biosystems Model 431A Peptide Synthesizer using the Fmoc/HBTU/HOBt program supplied with the instrument. The starting Fmoc-Cys(Trt)-HMP resin (polystyrene, 1% divinylbenzene copolymer) was purchased from Midwest Bio-Tech (0.412 g, 0.202 mmol). Amino acids were coupled as their N α -Fmoc derivatives. The side chains of Ser, Thr and Tyr were protected with the t-butyl group; those of Cys and Gln with the trityl group, those of Asp and Glu with the t-butyl ester group, that of Arg with the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC) group and that of Lys with the N ϵ -t-butyloxycarbonyl (t-Boc) group. Fmoc groups were removed at each cycle by a 6 min treatment with 20% piperidine in NMP. Each amino acid derivative was double coupled (25 min, room temperature), in 5-fold molar excess, using one equivalent of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) in NMP and DIEA. After the final coupling, the N-terminal Fmoc group was removed as described above and the resulting resin was washed with NMP and treated twice with 1-acetyl imidazole (550 mg, 5.0 mmol) in NMP (8 mL) for 25 min. The resulting 54 amino acid peptidyl-resin was washed with NMP and DCM and dried, yielding 1.471 g of B. Weight gain: 68% of theory.

25

30

Simultaneous deprotection and cleavage of the peptide (intermediate B) from the resin was accomplished by stirring the protected polypeptidyl-resin (1.471 g, 0.137 mmol) in 22 mL of CF₃COOH/water/thioanisole/dithiothreitol/phenol (10:0.5:0.5:0.3:0.8, v/v/v/w/w) for 3 hours at room temperature. The resin was

removed by filtration and the filtrate was concentrated in vacuo. The crude product was precipitated with cold methyl t-butyl ether (MTBE), filtered and washed with MTBE, then redissolved in 0.1% aqueous TFA and lyophilized. Yield: 0.645 g (77% of theory).

- 5 A portion of the crude linear product (140 mg) was stirred in 140 mL of 0.1 M NH_4HCO_3 /DMSO (9:1, v:v) for 20 hours at room temperature. The solution was lyophilized. Four sample aliquots (20 mg each) were injected into a Vydac C18 column (22X250 mm, 10 μ , 300 Å) and reverse phase HPLC was performed under the following conditions: Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in
- 10 CH_3CN ; linear gradient from 40 to 43% B in A over 50 min; flow rate: 12 mL/min. The fractions containing the major peak (215 nm) eluting between 27 and 30 min were pooled and lyophilized to yield 16 mg (20%) of Example 2 as a white, fluffy solid.
- 15 Analytical HPLC of the above-prepared OBF: Vydac C18 (4.6 x 250 mm); 215 nm, 1.0 mL/min: linear gradient from 35% to 45% B in A over 50 minutes; retention time 30.63 minutes. Purity: greater than 95% (Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in acetonitrile). MS (electrospray): found molecular weight (derived) 5788.6. Amino acid analysis: Asp(3) 3.2; Glx(10) 10.9; Ser(10) 8.7; Gly(3) 3.3; Arg(1) 1.1; Thr(2) 1.9; Ala(2) 1.9; Pro (3) 3.4; Tyr(1) 0.9; Val(4) 3.5; Cys(2) not quantitated; Ile(2) 1.4; Leu(10) 9.7; Lys(2) 2.2.
- 20 The above-prepared OBF gave a negative Ellman's test for free sulfhydryl.

Example 3

Process of Preparation of OBFs by Recombinant DNA Methods

25

Expression of OBFs in *Escherichia coli*

1. Method using PCR techniques

- 30 The OBFs can be expressed in *E. coli* as maltose binding fusion proteins (MBP) using an expression vector from New England Biolabs (pMALTM-c, 1990). The OBFs are amplified by PCR and cloned as Asp718/Sal I fragments by replacing most of the multiple cloning site and the factor Xa site of this vector. A two-step PCR protocol is used to simultaneously introduce a protease site (thrombin site) and a unique restriction enzyme site virtually anywhere within the OBFs.
- 35 As an example, a thrombin site downstream of Asn 99 and simultaneously a BamHI restriction enzyme site (bold) are introduced, resulting in the following construction:

95 99 100
 L Q I A N D L
 5 102
 E N (SEQ.IN.NO.:10)
 CTG CAG ATA GCC AAT CTG GTT CCG CGT **GGA TCC** GAC CTG
 GAG AAT (SEQ.ID.NO.:9)

10 The DNA and protein sequences of the *ob* gene construct containing a
 thrombin site within the gene are given in the one-letter code. The numbering refers to
 the amino acid numbering of the *ob* protein. Bold is the recognition sequence for the
 BamHI restriction enzyme. The DNA sequence in *italic* derives from the *ob* gene.

15 This product can be achieved with a two-step PCR reaction executed as follows:
 step 1a: template full length *ob* gene
 primer MAspNN and Nov2rev
 step 1b: template full length *ob* gene
 primer Nov2for and MSalCC
 20 step 2: templates products of PCR reactions 1a and 1b
 primers MAspNN and MSalCC

The following oligonucleotide sequences are used:

25 MAspNN: 5' - TCG GTA CCG GTG CCT ATC CAG AAA
 (SEQ.IN.NO.:11)
 MSalCC: 5' - GTC GAC TCA GCA TTC AGG GCT AAC ATC
 (SEQ.ID.NO.:12)
 Nov2rev: 5' - GAG ATT CTC CAG GTC GGA TCC ACG CGG AAC
 30 CAG ATT GGC TAT CTG CAG (SEQ.ID.NO.13)
 Nov2for: 5' - CTG CAG ATA GCC AAT CTG GTT CCG CGT GGA
 TCC GAC CTG GAG AAT CTC (SEQ.ID.NO.:14)

35 This system can be used for any modification within the gene by just changing the
 matching sequences in oligonucleotides Nov2rev and Nov2for.

2. Method using in vitro mutagenesis systems

A second strategy to obtain the construct shown in figure 1 is as follows. The *ob*
 gene is amplified as BamHI/Sal I fragment (using oligonucleotides MBamNN 5'-
 40 GGATCC GTGCCTATCCAGAAA GTC (SEQ.ID.NO.:15) and MSalCC 5'-
 GTCGAC TCAGCATTCAGGGCTAACATC (SEQ.ID.NO.:12)) and cloned into a
 mutagenesis vector system (i.e. pALTER-1 from Promega, 1994). The in vitro

- mutagenesis is performed as described by the manufacturer using the Nov2for oligonucleotide (see above). Finally, the mutated ob gene is cloned into the pMALTM c expression vector (New England Biolabs, 1990). This mutagenesis system also allows introduction of a thrombin site into the ob gene at any other location by
- 5 changing the matching sequences in oligonucleotide Nov2for.

Expression and purification of ob fragments expressed as maltose binding protein fusion in *E. coli*:

- 10 Expression:
medium: M9 minimal medium containing 0.5% amino acids,
trace metals, and 50 µg/ml ampicillin bacterial strain BL21;
expression conditions: induce bacteria in exponential growth phase (OD₆₀₀=0.8) with
0.5 mM IPTG shaking at 37 °C for 3-4 hours, collect cells by centrifugation (3500g)
15 and wash pellet once with TNE (10 mM Tris/HCl pH 8.0, 100 mM NaCl, 10 mM
EDTA),
shock freeze cell pellets in liquid nitrogen and store at -20 °C until use.

Purification:

- 20 The cell pellet (from 1 to 1.5 L cell culture) is resuspended in 30 mL lysis buffer (10 mM MOPS/NaOH pH 6.0, 2 mM EDTA, 500 mM NaCl, protease inhibitors) in a homogenizer and lysed by french press (SLM Aminco 40k preparative cell, single run at 1200 PSI). The lysis suspension is centrifuged (35 min, 35,000 rpm, Beckman 45TI rotor). The fusion protein is in the supernatant and can be directly affinity purified on
25 800-21 amylose resin (Biolabs Inc.) following the manufacturer's instructions. The protein is eluted in the lysis buffer containing 50 mM maltose. The pH of the eluate is increased to pH 8-9 and bovine thrombin (Amour Pharmaceutical Compny) is added to 5 U/mg fusion protein. The reaction is incubated for 30-40 min at RT and stopped with 1 mM benzamidine. The OBFs are precipitated with 40% acetone, oxidized and
30 further purified by reversed phase HPLC as described above.

Example 4 Treatment of ob/ob mice with OBF

- 35 **Animals:** Female obese (*ob/ob*) mice were obtained from Jackson Laboratory (Bar Harbor, ME) at 5-6 weeks of age. The animals were housed two per cage at

24°C with a 6 am to 6 pm light cycle. They were give ad lib access to water and powdered food (Purina 9F-5020) in food cups. Mice were acclimated for two weeks prior to implantation of the Alzet pumps.

- 5 **Implantation of Alzet pumps:** At day 0 of the study mice (n=6/group) were implanted with Alzet Model No. 2002 mini-osmotic pumps (Alza Corporation, Palo Alto, CA) containing OBF of SEQ.ID.NO.:2 as prepared in Example 2 (54-mer cyclized ob peptide) or an unrelated polypeptide of sequence: Leu-Ala-Arg-Ser-Leu-Ala-Pro-Ala-Glu-Val-Pro-Lys-Gly-Asp-Arg-Thr-Ala-Gly-Ser-Pro-Pro-Arg-Thr-Ile-Ser-
10 Pro-Tyr (control peptide, SEQ.ID.NO.:16). The pumps were implanted subcutaneously into the back area of each animal, under isoflurane anesthesia. Animals were infused with each peptide at a rate of 5 µg/mouse/day for 14 days.

- Evaluation:** Food consumption for each pair of mice was monitored daily and body
15 weight was taken at day 0, 7 and 13 of treatment. Animals were sacrificed at day 14, following a 16 hr fast, and plasma and abdominal white adipose tissue (WAT) were collected from each animal for analysis. WAT was quantitatively removed from each animal and immediately frozen in liquid nitrogen, and stored at -70°C.

- 20 Plasma chemistries were determined enzymatically using a Roche COBAS-MIRA S clinical analyzer. Plasma glucose and triglyceride levels were determined by an assay from Roche Diagnostics, Montclair, NJ; plasma non-esterified fatty acids (NEFA) levels were quantitated by an assay obtained from Wako Biochemicals, Wako, TX. Plasma insulin levels were determined by a radioimmunoassay (Incstar
25 Corporation, Stillwater, MN), utilizing rat insulin as the standard. Plasma corticosterone levels were determined by a double antibody radioimmunoassay (ICN Biochemicals, Costa Mesa, CA) utilizing rat corticosterone as the standard.

- Results:** No significant effect on food consumption, body weight gains, adipose
30 tissue mass, or plasma insulin and corticosterone levels (Table 1) were found in the group treated with OBF, relative to the control peptide. However, plasma glucose levels were significantly decreased by 34%, and plasma triglyceride levels were decreased by 20%, following 14 days of infusion with OBF, relative to infusion with the control peptide. Further experiments at similar doses of OBF infusion through
35 Alzet pumps showed non-statistically significant effects on plasma glucose and triglyceride levels. These results may be due to either insufficiently high dosage of

OBF or to substantial delivery of OBF to the intrascapular brown adipose tissue (BAT), possibly resulting from the subcutaneous placement of the Alzet pumps into the back of the animals. It is well documented that BAT is defective in ob/ob mice.

5 **Table 1. The Effect of Treatment of *ob/ob* Mice with polypeptides of the formula SEQ. ID. NO 1 (5 µg/day x 14 days, Alzet pump, s.c.)**

Parameter	Control	Polypeptide of the formula SEQ. ID. NO 1
Change in Body Weight (g)	4.7 ± 0.6	3.1 ± 0.9
WAT Mass (g)	5.8 ± 0.2	4.8 ± 0.1*** (-16%)
Plasma Glucose (mg/dl)	220 ± 20	146 ± 13** (-34%)
Plasma TG (mg/dl)	101 ± 4	81 ± 6* (-20%)
Plasma NEFA (mEq/L)	1.15 ± 0.06	1.36 ± 0.02** (+18%)
Plasma Insulin (ng/ml)	3.49 ± 0.39	4.56 ± 0.86
Plasma Corticosterone (ng/ml)	476 ± 49	516 ± 24

Data expressed as the mean ± SEM (n=6/group)

10 The statistical analysis is based on a two-tailed Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

The effects of intra-cerebroventricular (ICV) administration of OBF on food intake and body weight in obese (ob/ob) mice were non-statistically significant
15 which is believed to be due to sub-optimal dosage.

What Is Claimed Is:

1. A biologically active C-terminal fragment of *obese* gene product having less than 68 total amino acids, two of which are capable of forming cross-linkages.
- 5
2. A biologically active polypeptide having the sequence of SEQ.ID.NO.:1, SEQ.ID.NO.:2., SEQ.ID.NO.:3, SEQ.ID.NO.:4, or a polypeptide having at least 30% homology to any of said sequences; wherein said polypeptide has the following features: (1) residues in positions 117 and 167 with side-chains capable of forming cross-linkages ; (2) an amphipathic alpha-helix spanning about 15-25 C-terminal residues; and, (3) a loop supported by at least one helical scaffold and at least one covalent cross-linkage.
- 10
3. The polypeptide of Claim 2 having an amphipathic alpha-helix N-terminal to residue 117, spanning about 15-25 residues; and wherein the residues capable of forming cross-linkages are selected from the group consisting of disulfide, lactam, lactone, and dicarba-cystine.
- 15
4. The polypeptide of Claim 3 wherein residues 117 and 167 are cysteine.
- 20
5. A polypeptide having the sequence of SEQ.ID.NO.:1.
6. A polypeptide having the sequence of SEQ.ID.NO.:2.
- 25
7. A polypeptide having the sequence of SEQ.ID.NO.:3.
8. A polypeptide having the sequence of SEQ.ID.NO.:4.
9. A method for treating diabetes, obesity, or both in a patient in need of treatment, where the diabetes is *obese* protein-associated, the method comprising administering an effective amount of OBF to the patient.
- 30
10. The method of Claim 9 wherein said effective amount is about 0.001 to about 1000 mg per kg of body wt per day.
- 35

11. The method of Claim 9 wherein said effective amount is about 0.05 to about 50 mg per kg of body wt per day.

5 12. The method of Claim 9 wherein a concomitant decrease in food consumption does not occur.

13. A nucleic acid coding for the polypeptide of Claim 1.

10 14. The nucleic acid sequence of Claim 13 which is DNA.

15. A nucleic acid coding for the amino acid sequence of SEQ.ID.NO.:1, SEQ.ID.NO.:2, SEQ.ID.NO.:3, or SEQ.ID.NO.:4.

15 16. The nucleic acid sequence of Claim 15 which is DNA.

17. A DNA sequence having the sequence of SEQ.ID.NO.:5, SEQ.ID.NO.:6, SEQ.IN.NO.:7, or SEQ.ID.NO.:8, or a DNA sequence capable of hybridizing under stringent conditions to a DNA sequence complementary to any of said sequences.
20

18. A plasmid comprising the nucleic acid sequence of Claim 17.

19. A host cell containing the plasmid of Claim 18.

25 20. A method for producing the polypeptide of Claim 1 comprising culturing the host cell of Claim 18 under conditions suitable for expression of said polypeptide.

21. A method of diagnosing obesity or diabetes or a predisposition to develop obesity or diabetes in an individual, said method comprising

- 30 (a) labelling OBF;
 (b) contacting the labelled OBF with biological material from said individual;
 (c) monitoring the biological material for the presence of bound OBF to obtain a first set of results;

- 5
- (d) comparing the first set of results from step (c) with with a second set of results obtained using similar monitoring in an individual not having OBF-associated obesity or diabetes; and
 - (e) diagnosing the presence of obesity or diabetes in said individual or the tendency of said individual to develop obesity or diabetes by comparing the first set of results and second set of results to determine if the results differ.

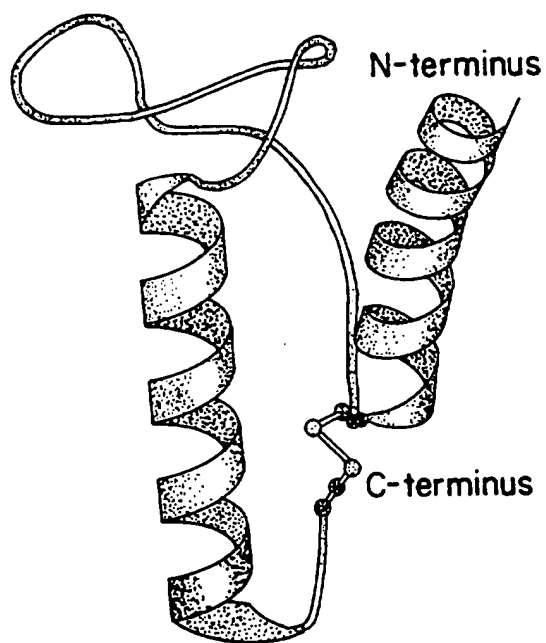


FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/17365

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C07K14/575 C12N1/21 G01N33/68 A61K38/22
//(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 23815 A (LILLY CO ELI ;HEATH WILLIAM F JR (US); MANETTA JOSEPH V (US); SHIE) 8 August 1996 see SepID's 3, 6, 18, 19, 22, 23 ---	1-4,8
P,X	WO 96 23514 A (LILLY CO ELI ;BASINSKI MARGRET B (US); DIMARCHI RICHARD D (US); FL) 8 August 1996 see SeqID's 11, 12, 14, 15 see claims; example 3 ---	1-4, 9-14, 17-20
P,X	WO 96 05309 A (UNIV ROCKEFELLER ;FRIEDMAN JEFFREY M (US); ZHANG YIYING (US); PROE) 22 February 1996 see page 36, line 18 - line 24 see page 55, line 1 - page 63, line 3 see page 75, line 15 - page 86, line 2; claims; examples ---	1,9-13, 18-20
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

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17.03.97

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/17365

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>NATURE, vol. 372, no. 6505, 1 December 1994, pages 425-432, XP000602062 YIYING ZHANG ET AL: "POSITIONAL CLONING OF THE MOUSE OBESE GENE AND ITS HUMAN HOMOLOGUE" cited in the application -----</p>	1-20

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-12
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9 to 12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds/compositions.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9623815 A	08-08-96	AU 4766596 A	21-08-96
WO 9623514 A	08-08-96	US 5525705 A	11-06-96
		US 5567678 A	22-10-96
		US 5580954 A	03-12-96
		US 5563244 A	08-10-96
		US 5532336 A	02-07-96
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		US 5563243 A	08-10-96
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		DE 19531931 A	07-03-96
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		ZA 9506868 A	09-04-96